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Sequence-Specific DNA Binding by the αNAC Coactivator Is Required for Potentiation of c-Jun-Dependent Transcription of the Osteocalcin Gene

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Since the c-Jun coactivator αNAC was initially identified in a differential screen for genes expressed in differentiated osteoblasts, we examined whether the osteocalcin gene, a specific marker of terminal osteoblastic differentiation, could be a natural target for the coactivating function of αNAC. We had also previously shown that αNAC can specifically bind DNA in vitro, but it remained unclear whether the DNA-binding function of αNAC is expressed in vivo or if it is required for coactivation. We have identified an αNAC binding site within the murine osteocalcin gene proximal promoter region and demonstrated that recombinant αNAC or αNAC from ROS17/2.8 nuclear extracts can specifically bind this element. Using transient transfection assays, we have shown that αNAC specifically potentiated the c-Jun-dependent transcription of the osteocalcin promoter and that this activity specifically required the DNA-binding domain of αNAC. Chromatin immunoprecipitation confirmed that αNAC occupies its binding site on the osteocalcin promoter in living osteoblastic cells expressing osteocalcin. Inhibition of the expression of endogenous αNAC in osteoblastic cells by use of RNA interference provoked a decrease in osteocalcin gene transcription. Our results show that the osteocalcin gene is a target for the αNAC coactivating function, and we propose that αNAC is specifically targeted to the osteocalcin promoter through its DNA-binding activity as a means to achieve increased specificity in gene transcription.

The calcium-binding osteocalcin protein is a terminal differentiation marker of the osteoblastic lineage. Dissection of the regulatory sequences controlling the osteoblast-specific expression of the osteocalcin gene has significantly improved our understanding of the molecular mechanisms regulating transcription during skeletogenesis, for example, through identification of Runx2/Cbfa1 as an osteoblast differentiation factor (22, 25). Characterization of osteocalcin gene transcription has uncovered several regulatory elements in the osteocalcin promoter (26), including binding sites for the AP-1 family of transcription factors (2, 5, 16, 28, 33–35, 40, 41).

The AP-1 family member c-Jun interacts with coactivators to potentiate transcription. One such coactivator has been characterized as αNAC (30, 36–38). The αNAC protein, first identified as a regulator of protein translation (45), was subsequently shown to also function as a transcriptional coactivator by potentiating the activities of the chimeric Gal4-VP16 activator (47) and of c-Jun homodimers (30, 36–38). αNAC provides a protein bridge between c-Jun and the basal transcriptional machinery by contacting the general transcription factor TBP (47). This stabilizes the c-Jun dimers on their cognate response element and results in enhanced transcription rates (30). In the course of those studies, it was shown that αNAC can specifically bind DNA, although it does not act as a transcription factor (48). It remained unclear whether the DNA-binding function of αNAC is expressed or if it is masked in vivo and whether it is required for the coactivating activity of αNAC.

The targeting of coactivators to particular promoters through sequence-specific DNA binding is a means to achieve increased specificity in gene transcription. For example, the B-cell specificity of octamer promoters is due to the interaction of the ubiquitous Oct-1 or lymphoid-specific Oct-2 factors with the B-cell-specific coactivator Bob-1 (24). To prevent the widespread activation of any promoter recognized by Oct-1 or Oct-2, Bob-1 activity is restricted to particular sites by virtue of its sequence-specific DNA-binding activity (9, 18). Similarly, certain TAF coactivators have been shown to bind core promoter elements, including the initiator region and the downstream promoter element (17).

Considering that we cloned αNAC as a differentially expressed protein in differentiated osteoblasts (30, 47), we examined whether the osteocalcin gene, a specific marker of terminal osteoblastic differentiation, could be a natural target for the coactivating activity of αNAC. We have identified an αNAC binding site within the murine osteocalcin gene proximal promoter region and demonstrated that recombinant αNAC or αNAC from nuclear extracts of osteoblastic ROS17/2.8 cells can specifically bind this element. Using transient transfection assays, we have shown that αNAC potentiated the c-Jun-dependent transcription of the osteocalcin promoter and that this activity specifically required the DNA-binding domain of αNAC or the intact αNAC binding site within the promoter region. Using chromatin immunoprecipitation, we observed that αNAC occupied its cognate binding site on the osteocalcin promoter in differentiated osteoblastic cells that express osteocalcin. Finally, inhibition of endogenous αNAC expression by

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use of siRNAs affected osteocalcin promoter activity in osteoblastic cells. Our results show that the osteocalcin gene is a natural target for the αNAC coactivating function and that αNAC is specifically targeted to the osteocalcin promoter, presumably as a means to achieve increased specificity in gene transcription.

MATERIALS AND METHODS

Abbreviations. αNAC, nascent polypeptide associated complex and coactivator alpha; Runx2/Cbfa1, Runt family member ×2/Core-binding factor alpha 1; AP-1, activating protein 1; Oct, octamer-binding protein; Bob-1, B-cell Oct-binding protein 1; OCN, osteocalcin; mmp-9, matrix metalloproteinase 9; OC box 1, osteocalcin box 1; ChIP, chromatin immunoprecipitation; DBD, DNA-binding domain; siRNA, small interfering RNA; TBP, TATA-binding protein; TAF, TBP-associated factors; EMSA, electrophoretic mobility shift assay; TBE, Tris-borate-EDTA; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Expression vectors and reporter plasmids. The p1316OG2-Luc reporter construct (herein referred to as OCN-Luc) (13) and the pCMV-Osf2 expression vector (14) (for expression of full-length Runx2/Cbfa1) were generous gifts from Gérard Karsenty (Baylor College of Medicine, Houston, TX). The mmp-9 pGL3 reporter (MMP9-Luc), which contains the proximal 670 bp of the mmp-9 gene promoter driving luciferase (19), was provided by Shoukat Dedhar (University of British Columbia, Vancouver, British Columbia, Canada). To engineer the OCN-Luc ΔBDG reporter, p1316OG2-Luc was digested at a unique Bst-AP1 restriction site within the osteocalcin promoter. The plasmid was then digested with Bal-31 nuclease following the manufacturer's procedure (New England Biolabs, Pickering, Ontario, Canada). The Bal-31-digested blunt-ended DNA fragments were self-ligated and used to transform *Escherichia coli*. Clones in which the αNAC binding site (positions −35 to −45 relative to the transcription start site) was deleted (plasmid OCN-Luc ΔBDG; the deletion covered from −32 to −60) were confirmed by direct DNA sequencing. Site-specific mutagenesis of the αNAC binding site within the murine osteocalcin promoter fragment (GC ACgGgGTAG [lowercase indicates mutated residues]; OCN-Luc mut BDG reporter) was performed by NorClone Biotech Laboratories (London, Ontario, Canada) using inverse PCR. The expression vectors for the Flag epitope-tagged full-length αNAC (pSI-NAC-Flag) (36) and for full-length c-Jun (pCI-cJun) (38) have been described previously. The cDNA for the Δ69-80 deletion mutant (αNACΔ69-80) was obtained by PCR cloning and inserted in-frame into pSI-Flag (36) to yield the pSI-αNACΔ69-80-Flag expression vector.

Transfections. COS-7 African green monkey cells were transfected using Lipofectamine reagent as described previously (36). Transfections utilized 300 ng of pCi-cJun alone or in combination with 300 ng of pSI-NAC-Flag or pSI-αNACΔ69-80-Flag. In one series of transfections, pCi-cJun was replaced by pCMV-Osf2. One hundred nanograms of reporter plasmids was used, and 40 ng of pSV6tk-CAT (11) was added to each sample as a control of transfection efficiency. All transfections were conducted in triplicate, and values are reported as means ± standard errors of the means. Statistical analysis was performed using analysis of variance and the Tukey or Bonferroni posttests. *P* values of <0.05 were accepted as significant. The expression level of transfected proteins was controlled by immunoblotting following immunoprecipitation with anti-Flag or anti-c-Jun antibodies (38).

EMSA. Complementary oligonucleotides corresponding to the putative αNAC binding site within the murine osteocalcin proximal promoter region (5′-GAG A*GCACAGAGTAG*CCGA-3′, positions −49 to −32 relative to the transcription start site [the putative binding site is shown in italics]) were synthesized with an overhang, annealed, and labeled with ^{32}P-labeled deoxynucleoside triphosphates by Klenow fill-in using standard protocols (3). One probe (see Fig. 2C) covered the αNAC binding site and the OC box 1 of the osteocalcin promoter, positions −103 to −30 relative to the transcription start site. Competitions used unlabeled double-stranded oligonucleotides corresponding to the previously identified αNAC binding site (5′-GAGA*CGACACACAGG*CCGA-3′; 1× NAC) (48) or the mutated osteocalcin sequence (5′-GAGA*GCACgGgGTAG*CCGA-3′; mut).

The recombinant αNAC proteins (wild-type and Δ69-80) were purified using NEB's IMPACT system as described previously (38). ROS17/2.8 nuclear extracts were prepared following the technique of Dignam et al. (12) as detailed elsewhere (7). Purified, recombinant c-Jun protein was purchased from Promega (Madison, WI).

Recombinant proteins or nuclear extracts were incubated for 30 min at 4°C in 20 μl of binding buffer [100 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 500 mM NaCl, 2% NP-40, 10 mM dithiothreitol, 10 mM EDTA, 100 ng of poly(dI-dC), 30% glycerol]. Labeled probe (5,000 dpm) was added to the binding reaction mixture. For competition experiments, the binding reaction was incubated with excess unlabeled 1× NAC or mut oligonucleotides. For supershift assays, anti-αNAC antibody (48) or anti-c-Jun antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added to the binding reaction for 15 min prior to the addition of the labeled probe. The bound mixtures were size fractionated on a nondenaturing 6% polyacrylamide gel at 140 V for 4 h in 0.5× TBE buffer. The gels were subsequently dried and autoradiographed.

ChIP. MC3T3-E1 cells (44) were plated on 100-mm-diameter dishes for 14 or 21 days in mineralizing media (10 mM β-glycerophosphate and 50 μg/ml ascorbic acid). Cells were then cross-linked by adding formaldehyde (1%, 20 min) with gentle agitation. The cross-linking was stopped by the addition of glycine to a final concentration of 125 mM for 10 min. The cells were then washed three times with ice-cold phosphate-buffered saline, scraped, and collected by centrifugation in 1 ml phosphate-buffered saline plus protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The cell pellet was resuspended for 10 min on ice in lysis buffer {5 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 8.0, 85 mM KCl, and 0.5% NP-40, with protease inhibitors as described above}. The nuclei were pelleted by centrifugation (5,000 rpm, 5 min), resuspended in 1.5 ml of nuclear lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors), and incubated on ice for 10 min.

To reduce the length of the chromatin fragments to approximately 600 bp, the extract was sonicated with a Sonic Dismembrator (model 500, Fisher Scientific Ltd., Nepean, Ontario, Canada), using four 15-s pulses at 30% of maximal power. After centrifugation (12,000 × *g*, 10 min, 4°C), the supernatant containing the chromatin was collected. Cross-linked extracts were diluted fivefold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, protease inhibitors). Immunoprecipitations were performed with Sepharose-conjugated anti-Flag monoclonal antibody (Sigma-Aldrich, St. Louis, MO), anti-αNAC polyclonal antibody (48), anti-IgG-agarose (as a negative control), or anti-Runx2/Cbfa1 polyclonal antibody (Santa Cruz). The immune complexes (anti-αNAC and anti-Runx2) were recovered with the addition of 150 μl of protein A-Sepharose beads and a subsequent incubation for 3 h at 4°C with agitation. The complexes were washed for 5 min with 1 ml of each of the following solutions: low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and LiCl wash buffer (250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Finally, they were washed twice in 1× Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

The protein-DNA complexes were eluted by two incubations with 250 μl of elution buffer (100 mM $NaHCO_3$, 1% SDS) with agitation and then centrifuged (10,000 × *g* for 3 min). To reverse cross-linking, the supernatant was collected and incubated with 1 μl of 10-mg/ml RNaseA in 300 mM NaCl at 65°C for 4 h. Then, the proteins were digested with 200 μg of proteinase K per ml for 1 h at 45°C. The DNA was precipitated overnight with ethanol at −20°C. DNA fragments were purified with a QIAquick Spin instrument (QIAGEN Inc.-Canada, Mississauga, Ontario, Canada). The sequences of the primers used were as follows. For αNAC forward, 5′-AGGCAGCTGCAATCACCA-3′ (beginning at nucleotide −153 of the murine osteocalcin promoter sequence) was used, and for αNAC reverse, 5′-GCACCCTGCAGCATCCA-3′ (position −2) was used. For Runx2/Cbfa1, the primers used were as published by Shen et al. (42): forward, 5′-AAATGAGGACATTACTGAACACTCC-3′ (position −459); and reverse, 5′-CCAAAGGATGCTGTGGTTGGTGAT-3′ (position −118).

Northern blot assay. Total RNA was isolated with the Trizol reagent (Life Technologies Inc., Burlinton, Ontario, Canada), and Northern blots were performed using a standard methodology. The probe used was the 470-bp EcoRI-PstI fragment from the mouse osteocalcin cDNA (8).

RNA interference. A subclone of MC3T3-E1 preosteoblasts showing high differentiation/mineralization potential and stably transfected with 1.3 kb of the mouse osteocalcin gene promoter driving expression of luciferase (46), a generous gift of Renny T. Franceschi (University of Michigan), was cultured at confluence for 7 days in the presence of G418. The cells were then passaged onto gelatin-coated wells at 20,000 cells/well in 24-well plates. The following day, the cells were transfected with 100 nM of siRNA directed against αNAC (catalog number 156855; Ambion Inc., Austin, TX) using 2.5 μl of siPORT Amine (Ambion) according to the manufacturer's instructions. Control wells were treated with the siPORT reagent only or were transfected with siRNA control no. 1, a sequence that shows no homology to any inventoried gene in the mouse or human databases (Ambion). The transfection mixture was left on the cells for 8 h, and then 1 ml of complete medium was added. RNA was harvested at 32 h posttransfection using the RNAqueous-4PCR kit (Ambion). The RNA was re-

verse-transcribed into cDNA using a High-Capacity cDNA Archive Kit per the manufacturer's recommendations (Applied Biosystems, Foster City, CA). Real-time PCR amplification was performed on an Applied Biosystems 7700 instrument using the TaqMan Universal PCR Master Mix (Applied Biosystems) and specific TaqMan assays for αNAC, OCN, and GAPDH. The expression level of each mRNA was quantified by the $\Delta\Delta C_T$ method (User bulletin no. 2, ABI Prism 7700 sequence detection system) and normalized to GAPDH levels. For luciferase activity, cell lysates were prepared in the reporter gene assay lysis buffer (Roche Molecular Biochemicals, Laval, Quebec, Canada). One hundred microliters of cell lysate was used for single luciferase reporter assays following the manufacturer's instructions (Promega). Luciferase activity was measured with a Sirius luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany). The expression level detected in cells treated with the transfection reagent alone was arbitrarily given a value of 1. Statistical significance was determined by analysis with an unpaired, one-tailed Student's *t* test for two-group comparison or by analysis of variance followed by the Dunnett's posttest for multiple group comparison. *P* values of <0.05 were accepted as significant.

RESULTS

αNAC specifically potentiates c-Jun-dependent transcription from the mouse osteocalcin promoter. Since we identified the c-Jun coactivator αNAC as a differentially expressed gene product in terminally differentiated mouse osteoblasts (30, 47), we tested whether the osteocalcin gene, a marker of the mature osteoblastic phenotype and an AP-1 target (2, 5, 16, 28, 33–35, 40, 41), would respond to the coactivating activity of αNAC. A reporter construct in which the luciferase gene was placed downstream from the murine osteocalcin gene promoter (13) was cotransfected with expression vectors for c-Jun and/or αNAC. Figure 1 shows that c-Jun activated transcription from the mouse osteocalcin gene promoter (bar 2) and that this effect was potentiated by cotransfection with full-length, wild-type αNAC (bar 4). Since it is a coactivator, not a transcription factor, expression of αNAC by itself did not influence reporter gene expression (bar 3), as previously observed with other promoters (36, 48). The coactivating activity of αNAC was specific to c-Jun, as cotransfecting αNAC with Runx2/Cbfa1, a key regulator of osteocalcin gene transcription (14, 22, 27), had no effect on Runx2/Cbfa1-mediated transcription (Fig. 1, bars 5 and 6). The expression of the transfected FLAG-NAC fusion protein was monitored by immunoblotting using the anti-αNAC antibody (48), which also detects the endogenous protein (Fig. 1B). Since each transfection was performed at least three times in triplicate with identical results, we are confident that the lack of coactivation activity of αNAC with Runx2/Cbfa1 was not due to the somewhat lower expression levels observed in the immunoblot shown in Fig. 1B. We conclude that αNAC specifically potentiates the c-Jun-mediated transcription of the osteocalcin gene.

αNAC specifically binds the osteocalcin proximal promoter region. We have previously reported that αNAC can bind the degenerate sequence 5′-C/G C/G A C/G A C/G A nnn G-3′, where n is any nucleotide (48). We identified a matching sequence between positions −35 and −45 of the mouse osteocalcin promoter (5′-GCACAGAgtaG-3′) and used EMSA to determine whether αNAC could specifically bind this element. A labeled, double-stranded probe corresponding to positions −49 to −32 relative to the osteocalcin transcription start site was synthesized and used in EMSA with purified recombinant αNAC. Figure 2A shows that the recombinant protein formed a strong complex with the labeled probe (lane 3) that was supershifted in the presence of a specific anti-αNAC antibody

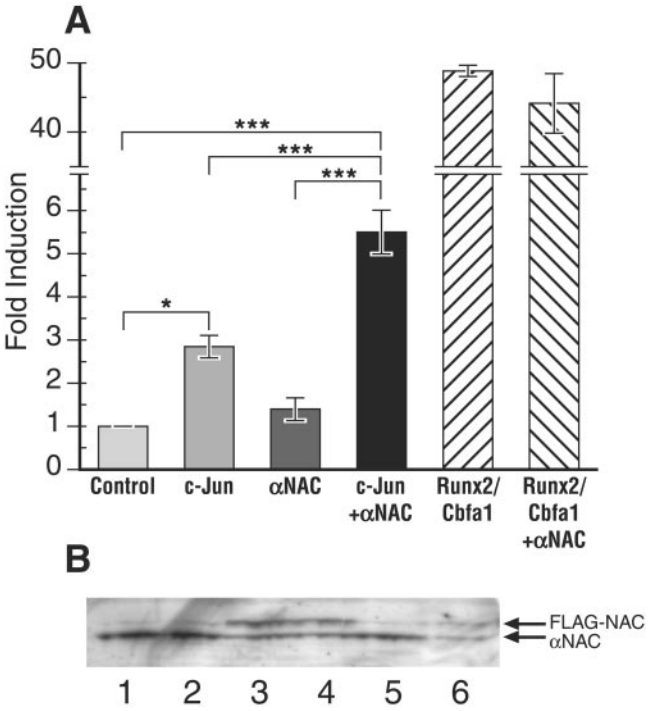

FIG. 1. αNAC specifically potentiates the transcription of the osteocalcin gene promoter by c-Jun. (A) COS-7 cells were transiently transfected with an osteocalcin-luciferase reporter and expression vectors for c-Jun, αNAC, or Runx2/Cbfa1, alone or in combinations. c-Jun induced the transcription of the reporter, and this induction was potentiated by αNAC. The coactivating function of αNAC was specific to c-Jun, as αNAC expression did not affect Runx2/Cbfa1-activated osteocalcin gene transcription. The expression level detected in cells transfected with the reporter construct alone was arbitrarily given a value of 1. Results are means ± standard errors of the means of three independent transfections performed in triplicate. *, $P < 0.05$; ***, $P < 0.001$. (B) Immunoblot probed with the anti-αNAC antibody that shows expression levels of endogenous and FLAG-tagged αNAC. The order of the numbered tracks corresponds to the order of the bars in panel A.

(lane 4) (48). A less intense complex with slower electrophoretic mobility that was not as affected by the antibody was also observed. This complex could be due to differentially phosphorylated forms of αNAC (36, 37). Preimmune serum or an excess of unlabeled mutated oligonucleotide did not influence binding (lanes 5, 6), while increasing amounts of an unlabeled oligonucleotide corresponding to the previously characterized αNAC binding site (48) efficiently competed binding of both complexes (lanes 7 to 9). An unrelated protein (maltose-binding protein) purified using the same system that was used for the purification of recombinant αNAC proteins did not bind the probe (lane 2), confirming that the binding activity was not due to a contaminant from the protein purification system. Nuclear extracts from the osteoblastic cell line ROS 17/2.8 also formed a complex with the labeled probe that was specifically but not completely supershifted by the anti-αNAC antibody, demonstrating that nuclear αNAC from osteoblasts could bind the osteocalcin sequence (Fig. 2B, lanes 2 to 4).

We have engineered a series of C-terminal and internal deletion mutants of the αNAC protein (37, 38) and tested them for DNA-binding activity with EMSA (data not shown).

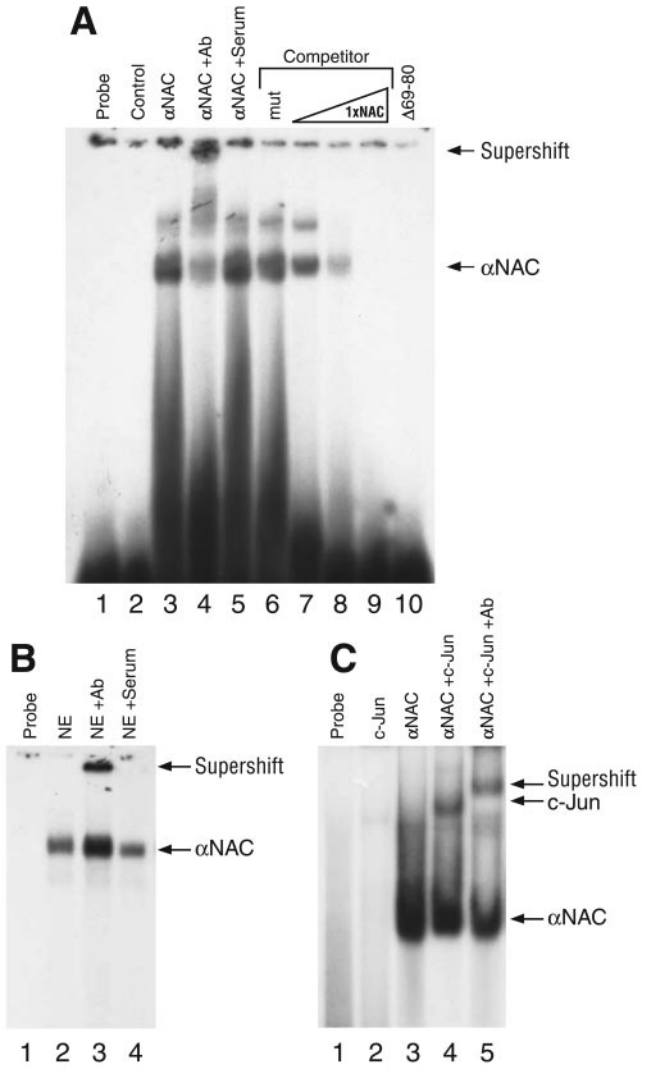

FIG. 2. αNAC and c-Jun bind the osteocalcin proximal promoter. EMSAs using a probe from the murine osteocalcin gene and purified recombinant αNAC (A), nuclear extracts (NE) from ROS 17/2.8 cells (B), or recombinant c-Jun and recombinant αNAC (C). In panel A, a specific complex can be detected (αNAC arrow) that was "supershifted" in the presence of anti-αNAC antibodies (Ab). Preimmune serum (Serum) did not influence complex migration. The complex was competed with increasing amounts of the canonical αNAC binding site (1xNAC) but not with a mutated sequence (mut). DBD-deleted αNAC (Δ69-80) did not bind the probe (lane 10). Panel B shows that αNAC from ROS 17/2.8 osteoblastic cells bound the probe. The complex was specifically but not completely supershifted by the anti-αNAC antibody (Ab). In panel C, the probe used covered positions −103 to −30 of the osteocalcin promoter and encompassed the αNAC binding site and the OC box 1 (34). Recombinant c-Jun did not bind the probe by itself (lane 2), but a complex containing c-Jun could be detected (c-Jun arrow) when the probe was incubated with both c-Jun and αNAC (lanes 4 and 5).

We found that deleting residues 69 to 80 of the 215-amino-acid αNAC protein abrogated the ability of the mutated protein (αNACΔ69-80) to bind DNA (Fig. 2A, lane 10). These experiments mapped the αNAC DNA-binding domain to residues 69 to 80 and showed that recombinant αNAC, as well as αNAC from osteoblastic nuclear extracts, could specifically bind the osteocalcin proximal promoter region in EMSA.

We also tested the binding of recombinant c-Jun to the mouse osteocalcin promoter. We used an oligonucleotide probe covering residues −103 to −30 of the mouse promoter. This probe included the αNAC binding site (−45 to −35) and a sequence that is 100% homologous to the OC box 1 from the rat osteocalcin gene promoter (−99 to −76) (13, 34). The rat OC box 1 was shown to bind Fos/Jun AP-1 complexes (34). As previously reported (34), c-Jun homodimers did not bind this element (Fig. 2C, lane 2). The probe efficiently bound recombinant αNAC (lane 3). Interestingly, when the probe was incubated with both αNAC and c-Jun, a novel complex formed (lane 4). This complex contained c-Jun, since it was supershifted by an anti-c-Jun antibody (lane 5). We interpret this result to mean that αNAC can stabilize the binding of c-Jun homodimers to the osteocalcin promoter, as was previously described for the AP-1 site from the metallothionein-IIA promoter (30).

αNAC must bind the osteocalcin promoter to coactivate c-Jun-dependent osteocalcin gene transcription. Transient transfection assays were used to determine the importance of the binding of αNAC to the osteocalcin promoter for its coactivating activity. In the first series of experiments, the activity of the DBD-deleted mutant (αNACΔ69-80) was compared to the activity of intact αNAC. Figure 3A shows that contrary to its wild-type counterpart, αNACΔ69-80 could not potentiate c-Jun-mediated osteocalcin gene transcription. Both αNAC and αNACΔ69-80 were efficiently expressed in those experiments (Fig. 3B, second panel). The FLAG-tagged αNAC proteins were expressed at levels similar to that of endogenous αNAC (Fig. 3B, third panel) (note that FLAG-αNACΔ69-80 comigrates with endogenous αNAC, and thus the anti-αNAC antibody recognizes both proteins at the same position in the gel).

The αNAC binding element was then mutated or deleted from the osteocalcin promoter. While c-Jun could still increase transcription from both mutated reporters (OCN-Luc ΔBDG and OCN-Luc mut BDG; Fig. 4A and B, respectively), wild-type αNAC could not potentiate c-Jun-dependent expression of the reporter gene when its binding site was mutated (Fig. 4B, bar 8) or removed from the osteocalcin promoter (Fig. 4A, bar 6). Taken together, those data show that αNAC must interact with the promoter to coactivate osteocalcin gene expression induced by c-Jun.

The αNAC DNA-binding domain is specifically required for osteocalcin expression. To determine whether αNAC must always bind DNA to exert its coactivating activity, we compared the activity of wild-type αNAC and the DBD mutant, αNACΔ69-80, on two natural target promoters, osteocalcin and mmp-9 (36). As described above, αNACΔ69-80 could not potentiate c-Jun-mediated transcription from the OCN-Luc template (Fig. 5). Transcription from the mmp-9 promoter is induced by c-Jun, and wild-type αNAC potentiates this activity (Fig. 5, bars 7 to 10) (36). Transfection of αNACΔ69-80 by itself led to an increase in MMP9-Luc expression. Interestingly, the DBD-deleted mutant of αNAC potently coactivated c-Jun-dependent transcription from the mmp-9 promoter (Fig. 5, bar 12). We interpret these data to mean that the αNAC DNA-binding activity is not always necessary for coactivation,

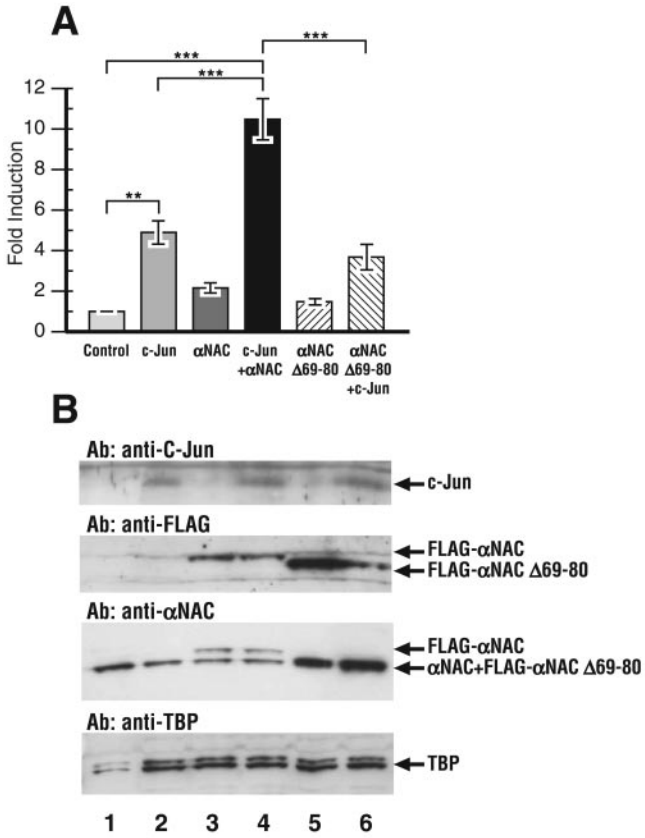

FIG. 3. Coactivation of c-Jun-dependent osteocalcin gene transcription requires the αNAC DBD. (A) Transient transfection assays were set up as described in the legend to Fig. 1. The recombinant αNACΔ69-80 protein, devoid of DNA-binding activity, did not potentiate the activity of c-Jun. **, $P < 0.01$; ***, $P < 0.001$. (B) The expression of the recombinant proteins was monitored by Western blot assay using the antibodies (Ab) listed above each panel. Probing with anti-TBP served as a loading control. Note that FLAG-αNACΔ69-80 migrates at the same position as endogenous αNAC in SDS-PAGE, so that the band detected by the anti-αNAC antibody (third panel, lanes 5 and 6) represents the combined signal of endogenous αNAC and FLAG-αNACΔ69-80.

but that it is a specific requirement for the potentiation of c-Jun-dependent transcription from the osteocalcin promoter.

The αNAC-DNA interaction occurs in vivo. We used chromatin immunoprecipitation to determine if αNAC occupies its cognate binding site on the osteocalcin promoter in living cells expressing osteocalcin. Wild-type osteoblastic MC3T3-E1 cells or MC3T3-E1 cells stably transfected with pSI-NAC-Flag or FLAG-αNACΔ69-80 were grown for 14 or 21 days postconfluence in the presence of ascorbic acid and beta-glycerophosphate. The expression of the wild-type or fusion proteins was monitored by immunoblotting (Fig. 6B). Note that FLAG-αNACΔ69-80 migrates at the same position as endogenous αNAC in SDS-PAGE, so that the band detected by the anti-αNAC antibody, shown in Fig. 6B, lane 3, represents the combined signal of endogenous αNAC and FLAG-αNACΔ69-80. Osteocalcin gene expression can readily be observed after 14 days in culture under the conditions used (15) (Fig. 6C). DNA was cross-linked to proteins using formaldehyde, and sonicated chromatin from whole-cell lysates was coimmunoprecipitated using specific or negative control antibodies. DNA fragments that coprecipitated with the target proteins were purified upon reversal of protein/DNA cross-links and used as templates for PCRs with osteocalcin-specific primers. As a positive control for the assay, we used antibodies directed against Runx2/Cbfa1 and previously published primer pairs (42). The 350-bp amplimer diagnostic of Runx2/Cbfa1 binding to the osteocalcin promoter (42) was readily observed (Fig. 6A and D, lanes 1 and 2).

Cross-linked DNA was then immunoprecipitated using anti-αNAC or anti-Flag antibodies and amplified using primer pairs that flank the αNAC binding site within the osteocalcin proximal promoter. The diagnostic 151-bp amplimer could be observed using both antibodies (Fig. 6A, lanes 5 to 8), and the amount of amplified DNA correlated with osteocalcin gene expression (Fig. 6A and C). No DNA was amplified when nonspecific IgGs were used for immunoprecipitation (Fig. 6A, lane 4). Similarly, the anti-Flag antibody did not immunopre-

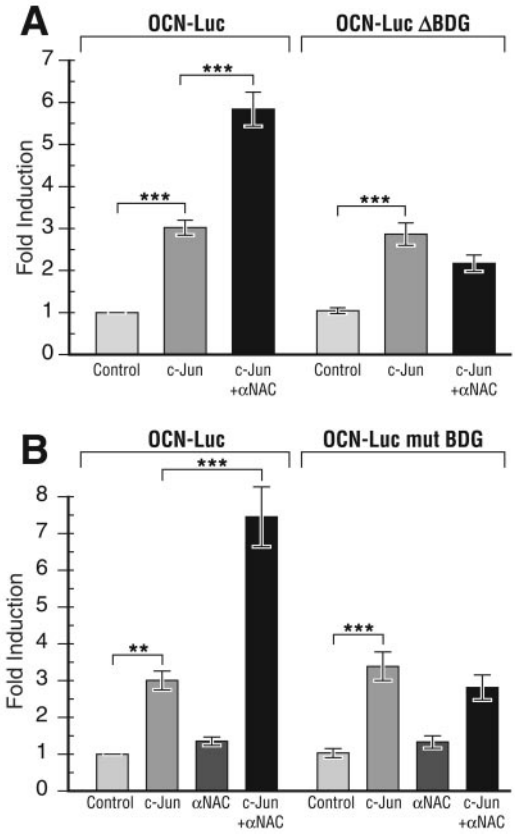

FIG. 4. Coactivation of c-Jun-dependent osteocalcin gene transcription requires the αNAC binding site on the promoter. Transient transfection assays were performed as described in the legend to Fig. 1. (A) Reporters used included the wild-type osteocalcin promoter driving luciferase (OCN-Luc) or a mutated osteocalcin promoter in which the αNAC binding site was deleted (OCN-Luc ΔBDG; the deletion covered from −32 to −60 relative to the transcription start site). (B) Reporters used included OCN-Luc and a mutated osteocalcin promoter in which the αNAC binding site was mutated by site-specific mutagenesis (OCN-Luc mut BDG; the engineered mutation was 5′-GCACgGgGTAG-3′). αNAC did not potentiate the activity of c-Jun when its binding site was mutated or deleted from the promoter. **, $P < 0.01$; ***, $P < 0.001$.

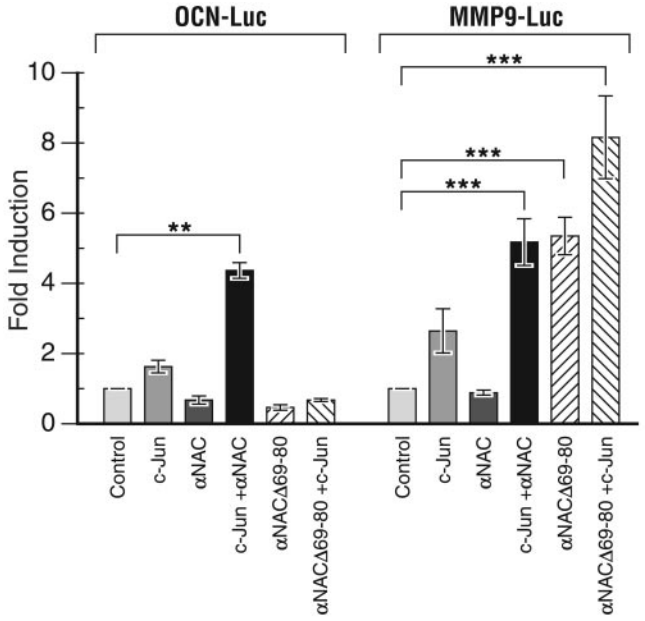

FIG. 5. αNAC DNA-binding activity is specifically required for c-Jun-dependent osteocalcin gene transcription. Transient transfection assays were set up as described in the legend to Fig. 1. Two c-Jun responsive promoters were used as reporter constructs: the wild-type osteocalcin promoter (OCN-Luc) or the proximal 670 bp of the mmp-9 gene promoter (MMP9-Luc). Wild-type αNAC could potentiate the activity of c-Jun on both promoters, but the DNA-binding domain-deleted αNAC mutant (αNACΔ69-80) was active only on the MMP9-Luc template. **, $P < 0.01$; ***, $P < 0.001$.

cipitate DNA from untransfected MC3T3-E1 cells at 21 days postconfluence (lane 9). As an additional negative control, primer pairs selected from the osteocalcin coding sequence were selected. These primers did not amplify chromatin immunoprecipitated with either the anti-αNAC or anti-Flag antibodies (data not shown).

MC3T3-E1 cells were also stably transfected with the Flag epitope-tagged DBD-deleted αNAC mutant, αNACΔ69-80 (Fig. 6B, lane 3). In those transfectants, an osteocalcin promoter fragment was coimmunoprecipitated with the endogenous wild-type αNAC (Fig. 6D, lane 3), while the anti-Flag antibody, which precipitated αNACΔ69-80 (not shown), did not coimmunoprecipitate osteocalcin chromatin (Fig. 6D, lane 4).

These data show that in living osteoblastic cells expressing osteocalcin, αNAC occupies its cognate binding element. They further confirm that the αNACΔ69-80 deletion mutant is devoid of DNA-binding activity.

Inhibition of endogenous αNAC expression affects osteocalcin promoter activity. To confirm the relevance of αNAC in the control of osteocalcin gene transcription, we inhibited endogenous αNAC expression using RNA interference. These experiments were performed using a subclone of MC3T3-E1 preosteoblasts exhibiting high differentiation/mineralization potential that has been stably transfected with a luciferase reporter gene under the control of the 1.3-kb mouse osteocalcin promoter fragment (46). The transcriptional control of this stably integrated reporter allele was shown to be identical to that of the endogenous gene (46). Specific siRNAs for αNAC

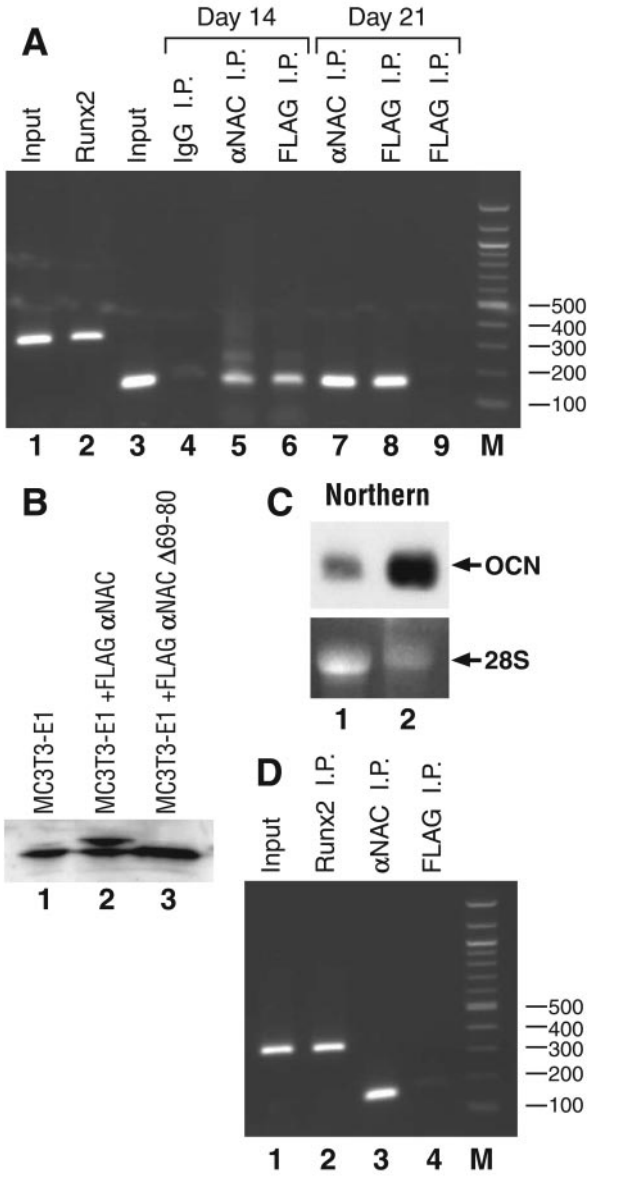

FIG. 6. αNAC binds the osteocalcin gene promoter in living cells expressing osteocalcin. Wild-type osteoblastic MC3T3-E1 cells or MC3T3-E1 cells stably transfected with pSI-NAC-Flag (A) or the Flag epitope-tagged DBD-deleted αNAC mutant, αNACΔ69-80 (D), were grown for 14 (panel A) or 21 (panels A and D) days postconfluence in the presence of ascorbic acid and beta-glycerophosphate. Immunoprecipitation assays were performed with formaldehyde-cross-linked chromatin and antibodies against Runx2/Cbfa1, αNAC, or the Flag epitope. Ethidium bromide-stained agarose gels of PCR products obtained with primers flanking the Runx2/Cbfa1 binding site (lanes 1 and 2) or the αNAC binding site (lanes 3 to 9) within the mouse osteocalcin gene promoter are shown. Input, amplification of DNA prior to immunoprecipitation; IgG, immunoglobulin G; I.P., immunoprecipitate; M, molecular size markers. (B) Immunoblot probed with the anti-αNAC antibody that shows expression levels of endogenous and FLAG-tagged αNAC proteins. Note that FLAG-αNACΔ69-80 migrates at the same position as endogenous αNAC in SDS-PAGE, so that the band detected by the anti-αNAC antibody (lane 3) represents the combined signal of endogenous αNAC and FLAG-αNACΔ69-80. (C) Northern blot showing OCN mRNA expression. 28S, ribosomal 28S RNA used to monitor loading.

inhibited 90% of the αNAC mRNA when quantified by real-time reverse transcription-PCR (Fig. 7A) and considerably reduced αNAC protein expression over the duration of the experiment (Fig. 7B). The efficiency of the αNAC siRNA in inhibiting its target was compared to that of an unrelated control siRNA (Fig. 7A and B, lane 2) or to the transfection reagent alone (Fig. 7B, lane 1). We next assessed the impact of αNAC siRNA knock-down on osteocalcin gene transcription. Treatment of cells with the control siRNA affected osteocalcin expression, which was lower in transfected cells than in untransfected controls (relative expression was calculated as 26%) (Fig. 7A and C). This could be due to the experimental conditions, which included the subculturing of confluent cells into sparse cultures combined with treatment with the transfection reagent. Nevertheless, treatment of cells with the specific αNAC siRNA that inhibited αNAC mRNA and protein expression further repressed both endogenous osteocalcin mRNA levels (Fig. 7A) and the transcription of the osteocalcin promoter-controlled luciferase reporter (Fig. 7C). The effect of the αNAC siRNA treatment on endogenous osteocalcin expression nearly reached statistical significance ($P = 0.0512$) (Fig. 7A), while its impact on the transcription of the reporter allele was highly significant (Fig. 7C). These data establish the physiological relevance of endogenous αNAC as a regulator of osteocalcin gene transcription in osteoblastic cells.

DISCUSSION

We have previously shown that αNAC functions as a c-Jun coactivator (30, 36–38). The cloning of αNAC as a differentially expressed gene product in terminally differentiated osteoblasts (47) and the expression of the αNAC protein in mineralizing osteoblasts at the ossification centers of developing embryos (30) suggested that αNAC should be involved in some aspects of the regulation of gene transcription in differentiated bone-forming cells. We have now identified the osteocalcin gene as a natural target gene for αNAC's coactivating function. Osteocalcin encodes a bone-specific protein induced in osteoblasts with the onset of mineralization at late stages of differentiation (32). Interestingly, we have found that the regulation of osteocalcin gene transcription by αNAC specifically required the previously identified DNA-binding activity of αNAC (48), and we have mapped the αNAC DNA-binding domain between amino acid residues 69 and 80. Confirming the in vitro assays, we showed that αNAC binds the osteocalcin proximal promoter region in differentiated osteoblastic cells expressing osteocalcin and that specific inhibition of αNAC by RNA interference affects osteocalcin promoter activity.

Functional AP-1 binding sites have been characterized for the human (16, 35, 41) and rat (2, 5, 28, 33, 34) osteocalcin promoters. Transcriptional induction of the murine osteocalcin promoter by AP-1 family members has also been reported (40). The precise mouse osteocalcin AP-1 response element remains to be characterized, but we speculated that regions from the mouse gene showing homology to characterized AP-1 elements in the promoter of the osteocalcin gene from other species could be functional sites. The most likely candidate sequence would correspond to the "OC box 1" between nucleotides −75 and −98, which is 100% homologous between rat and mouse. The rat "OC box 1" sequence was shown to bind AP-1 molecules (34). Interestingly, the αNAC binding site that

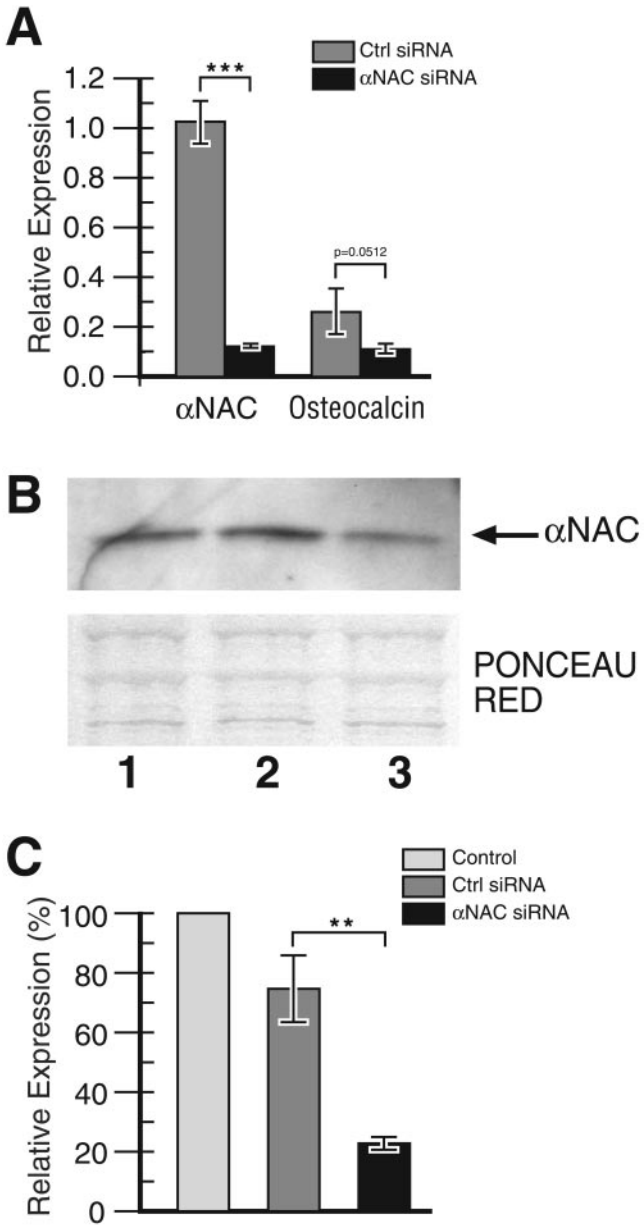

FIG. 7. Inhibition of endogenous αNAC expression by RNA interference affects osteocalcin promoter activity. MC3T3-E1 cells stably transfected with a luciferase reporter gene under the control of the 1.3-kb mouse osteocalcin promoter fragment (46) were transfected with a control, unrelated siRNA, or an siRNA directed against αNAC. (A) Expression of the endogenous αNAC or osteocalcin mRNAs was monitored by real-time PCR. ***, $P < 0.001$. (B) Expression of the endogenous αNAC protein was assessed by immunoblotting with the anti-αNAC antibody. Lane 1, treatment with the transfection reagent alone; lane 2, transfection with the control siRNA; lane 3, transfection with the αNAC siRNA. Staining of the membrane with Ponceau red (bottom panel) was used to monitor for even loading of each lane on the gel. (C) Expression of the reporter luciferase gene under the control of the osteocalcin promoter was monitored with a luminometer. The control bar represents expression measured in the presence of the transfection reagent alone. **, $P < 0.01$; Ctrl siRNA, control siRNA. Treatment of cells with the specific αNAC siRNA repressed both endogenous osteocalcin mRNA levels and the transcription of the osteocalcin promoter-controlled luciferase reporter.

we have identified is adjacent to "OC box 1." While recombinant c-Jun did not bind the OC box 1 by itself (Fig. 2C) (34), a binding complex that contained c-Jun was detected when the transcription factor and coactivator were incubated together with the probe (Fig. 2C). We propose that the binding of c-Jun to its response element on the osteocalcin promoter requires αNAC, which has been previously shown to stabilize the binding of c-Jun homodimers on canonical AP-1 sites (30). This model would explain why previous efforts to detect homodimeric c-Jun binding to the osteocalcin promoter were unsuccessful, since they did not include αNAC in the binding reaction. Further studies are under way to precisely delineate the c-Jun binding site and define the molecular determinants of the c-Jun-αNAC interaction on the mouse osteocalcin gene promoter.

In differentiated rat osteoblasts, Fra-2/JunD heterodimers were identified as the major AP-1 complexes (28, 29). Our results show that the mouse osteocalcin gene promoter can respond to c-Jun. It remains to be determined whether the transfected c-Jun protein dimerized with endogenous Fos family members to activate osteocalcin gene transcription. Dimerization with c-Fos appears unlikely, as we have previously shown that αNAC cannot potentiate the activity of the c-Fos/c-Jun heterodimer (30). Potential c-Jun dimerization partners would include Fra-1 or ΔFosB, which have been shown to play functional roles in osteoblasts in the regulation of bone mass accrual (21, 23, 39, 40, 43). We favor the possibility that the c-Jun homodimer regulates osteocalcin gene transcription and that this effect is maximized in the presence of the c-Jun coactivator, αNAC. This interpretation could be formally tested using the recently described expression vectors for single-chain tethered AP-1 dimers (4).

Our results confirm the previously reported specific DNA-binding activity of αNAC (48). This contrasts with published work claiming that the nucleic acid-binding activity of NAC subunits is nonspecific (6). These studies also utilized recombinant αNAC purified from bacteria, although they were purified with a different fusion protein system. We cannot find obvious reasons for the discrepancies between the results obtained in each case. The binding element that we identified herein within the osteocalcin promoter region matches the consensus sequence characterized with the PCR-based technique involving selection and amplification of the binding site (48). The αNAC binding site in the osteocalcin promoter is fairly conserved among species (see the promoter sequence comparisons in reference 13), but the functionality of the homologous sequences from the rat and human promoters remains to be tested. It will be interesting to determine whether other genes contain similar sequence elements within their regulatory regions, although preliminary data suggest that homologous sequences are rare within the available databases (not shown).

We observed that αNAC's DNA-binding activity was required for osteocalcin gene coactivation but was dispensable for the potentiation of c-Jun-dependent transcription of the mmp-9 gene. This raises the interesting possibility that αNAC may be targeted to the osteocalcin promoter to achieve an increased specificity for gene transcription in differentiated osteoblasts. Such a mechanism has already been described for the B-cell-specific coactivator Bob-1 (9, 18).

Chromatin immunoprecipitation demonstrated that αNAC occupied its cognate binding site in intact osteoblasts expressing osteocalcin. In nonexpressing cells, several mechanisms could operate. The coactivator might be prevented from contacting its binding element either by the inaccessibility of the chromatin or by posttranslational regulation. The first hypothesis is supported by the well-characterized role of chromatin-modifying complexes in the regulation of gene transcription (31). The second hypothesis is supported by studies showing that the activity of coactivators can be regulated by protein kinases. Phosphorylation of residue Ser184 is essential for inducible activation by the coactivator Bob-1 (49). CBP contains a signal-regulated domain required for stimulation of transcription; phosphorylation of this domain is controlled by nuclear calcium and calcium/calmodulin-dependent protein kinase IV (10, 20). The histone acetyltransferase activity of CBP is also regulated upon phosphorylation by p44 MAPK/ERK1 (1). We have shown that differential phosphorylation of αNAC controls its half-life (36) and its nuclear entry (37). It is possible that differential phosphorylation might regulate its DNA-binding activity. Indeed, computer-based analysis identifies residue Ser70 within the DNA-binding domain as a potential phosphoacceptor site (data not shown).

Alternatively, αNAC might occupy its cognate binding site even in nonexpressing cells. In this scenario, posttranslational modifications such as phosphorylation could function to allow αNAC to recruit transcriptional repressors that would maintain osteocalcin gene expression as silent. The induction of osteocalcin expression would require a different pattern of posttranslational modifications of the αNAC protein that could then function as a coactivator of osteocalcin transcription. Additional studies are required to distinguish between these various possibilities.

The osteocalcin gene promoter has been extensively studied. It is modularly organized and contains several positive and negative regulatory elements (26). Our results have identified yet another regulatory sequence within the proximal osteocalcin promoter region, the αNAC binding site. The down-regulation of osteocalcin promoter activity measured when endogenous αNAC levels are reduced in osteoblastic cells confirms the physiological relevance of αNAC in the control of osteocalcin gene transcription. It will prove interesting to characterize the functional interplay between the various transcriptional regulators that control osteocalcin promoter activity in differentiating osteoblasts.

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